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For the development of HER2/neu anti-idiotype antibodies, groups of female BALB/c mice were immunized with 454C11 monoclonal antibodies directed at HER2/neu. Hybridomas were generated by fusing spleen cells from immunized mice with non-secretory mouse myeloma cells. One Ab2 producing hybrid of 454C11 was obtained, cloned by limiting dilution but showed declining production of Ab2. The specificity binding study using purified 741F8 Ab2 and a series of mouse monoclonal antibodies belonging to different subclasses and directed against different antigens showed that the binding of 741F8 Ab2 was to those monoclonal antibodies directed against HER/2neu. A competitive binding assay using 741F8 Ab2, Ab1 and the human breast cancer cell line SK-BR-3 (which express HER2/neu on their cell surface), showed that as little as ~10ng of purified 741F8 Ab2 inhibited the binding of Ab1 to SK-BR-3 cells by ~50%. Rabbits and mice have been immunized with purified 741F8 Ab2 to generate an Ab3 response.

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Malaya Bhattacharya-Chatterjee July 15, 1996

PI - Signature Date

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INTRODUCTION

Breast cancer is a major cause of cancer deaths in women. The incidence of breast cancer has steadily increased over the last two decades and patients with recurrent disease are not curable by standard therapies. In human breast cancer, amplification and overexpression of the cell membrane protein HER2/neu, which is not present on normal breast tissue, has been observed to occur in a significant number of tumors. In 189 primary human breast cancers, HER2/neu was found to be amplified from 2 to over 20 fold in 30% of the tumors (1). Patients with multiple copies of the HER2/neu gene in DNA from their tumors had a shorter time to relapse as well as a shorter overall survival (1-3), indicating that HER2/neu gene amplification was prognostic for both disease behavior and clinical outcome in these patients. Not only were increased copy numbers of HER2/neu in breast cancers related to a poorer prognosis, but gene amplification of HER2/neu correlated with lymph node involvement (1-5), histological grade (5,6), negative estrogen receptor content (7,8), early recurrence (4,7), increased mitotic activity (9), all of which are considered to be poor prognostic indicators. In a retrospective study, the expression of HER2/neu determined immunohistochemically in positive breast cancer samples from 253 patients found that HER2/neu positive breast cancers behaved more aggressively in the first 2-3 years following diagnosis (10). Several studies have shown that overexpression of HER2/neu occurs in as many as 15-40% of breast cancers and that overexpression of HER2/neu is associated with poor survival (1-3). Therefore HER2/neu present on the surface of overexpressing breast cancer cells offers a good target for immunotherapy. It has been shown that monoclonal antibodies raised against the human protein that bind to the extracellular domain of HER2/neu can inhibit the growth of tumor cells *in vitro* (11-13) and *in vivo* (14). A recent report has determined that breast cancer specific cytotoxic T lymphocytes recognize a 9 amino acid peptide from the transmembrane portion of the HER2/neu protein (15).

Since cancer patients are often immunosuppressed and also tolerant to some tumor-associated antigens such as HER2/neu, triggering an active immune response to such antigens represents a challenge in cancer therapy. One approach has been to use tumor derived material as the immunogen. As an alternative to the use of tumor antigens or tumor cells, the network hypothesis of Neils Jerne (16) offers a different approach to vaccine therapy using the so-called internal image antigens (17-21). According to the network concept, immunization with a given antigen will generate the production of antibodies against this antigen termed Ab1. This Ab1 can generate a

series of anti-idiotype antibodies termed Ab2. Some of these Ab2 molecules can effectively mimic the three dimensional structures of external antigens. These particular anti-idiotypes called Ab2ß, which fit into the paratopes of Ab1, can induce specific immune responses similar to the nominal antigen. Anti-idiotype antibodies of the ß type express the internal image of the antigen recognized by the Ab1 antibody and can be used as surrogate antigens. Immunization with Ab2B can lead to the generation of anti-anti-idiotype antibodies (Ab3) that recognize the corresponding original antigen. Indeed, human trials using anti-idiotype monoclonal antibodies (Ab2ß) to stimulate immunity against the patients own tumor has shown promising results. Objective clinical improvement has been observed in patients with colorectal cancer, melanoma and T-cell lymphoma (for reviews see 22,23).

Our overall aim has been to apply this approach to the treatment of breast cancer by attempting to develop monoclonal anti-idiotype antibodies against HER2/neu starting with a series of mouse monoclonal antibodies (Ab1) that recognize different epitopes of HER2/neu (24-26). We selected 3 monoclonal antibodies 520C9, 741F8 and 454C11 which recognize distinct antigenic determinants on HER2/neu. These monoclonal antibodies appear highly selective; immunoperoxidase staining of normal human tissues showed negligible staining with these antibodies (26). Furthermore, when conjugated to ricin A, these antibodies produced immunotoxins selectively cytotoxic to SK-BR-3 breast cancer cells (26). The restricted specificity of these monoclonal antibodies together with their high binding capacity to a representative breast carcinoma cell line SK-BR-3, make them excellent target for generating Ab2 hybridomas. The use of various Ab2 that mimic multiple epitopes on the tumor cell surface may increase immunotherapeutic efficacy of Ab2 immunizations. Furthermore, the internal image antigens (Ab2) generated against these 3 monoclonal antibodies may cover almost all of breast tumors expressing HER2/neu and in many cases will complement each other.
Our overall goal is to develop monoclonal anti-idiotype antibodies as a surrogate for the tumor associated antigen HER2/neu to be used for the treatment of breast cancer.

BODY

Materials and Methods

Animals

Female BALB/c mice, 6-8 weeks old and male New Zealand rabbits 4-6 months old from Harlan Laboratories.

Cells

Established human cell lines were obtained from the American Type Culture Collection (Rockville, MD). SK-BR-3 cells were cultured in Mc Coy's Medium and HBL 100 cells cultured in RPMI 1640 each supplemented with 10% fetal calf serum.

Antibody

Mouse monoclonal antibodies 741F8 and 454C11 were obtained from Chiron Corp. These antibodies were used to immunize female BALB/c mice for the production of anti-idiotype antibodies.

Immunization of BALB/c mice

Groups of BALB/c mice, 6-8 weeks old were immunized with monoclonal antibody 454C11 (Ab1). The first injection was administered i.p. with 100μg of Ab1 mixed with Freund's complete adjuvant. The second injection was administered s.c. two weeks later with 100μg of Ab1 in Freund's incomplete adjuvant. Subsequent inections were given i.p. at two week intervals, with 100μg of Ab1 coupled to KLH. Mice were bled two weeks following each injection and the sera checked for anti-idiotype activity by Sandwich RIA using the respective Ab1. Mouse IgG1 was used as control. Three days prior to the fusion, mice were boosted with Ab1 (100μg in PBS) injected intravenously into the tail vein.

Coupling of antibody with keyhole limpet hemocyanin (KLH)

Antibody stock solution (1mg/ml) was mixed with KLH (1mg/ml) in PBS in the presence of glutaraldehyde (0.05%). The mixture was rotated for 2 hours at room temperature and then dialyzed for 24 hours against PBS in the cold (27).

Fusion

Fusion of BALB/c mouse spleen cells with non-secretory P3-653 myeloma cells using 50% polyethyleneglycol (3,400 mwt. Aldrich Chemical Co., Milwaukee, WI) was carried out as previously described (27-29). Hybrids were selected using hypoxanthine-aminopterine-thymidine media.

Selection of 454C11 anti-idiotype antibodies (Ab2)

Initial screening of hybridomas for anti-idiotype antibody production was done by Sandwich RIA using the respective Ab1 at a concentration of 250 ng/well to coat plates (27,28). After overnight incubation at 4°C, plates were blocked with 1% bovine serum albumin in PBS for 30 minutes. Thereafter, 50 μl of undiluted hybridoma culture supernatents were incubated for 2 hours at room temperature with continuous shaking. After washing with PBS, plates were further incubated for 2 hours at room temperature with ~50,000 cpm of I-125-radiolabelled Ab1. Ab2 producing fusion wells were expanded in hypoxanthine-thymidine media and cloned by limited dilution to obtain a single

population of Ab2 producing cells using monolayers of mouse peritoneal macrophages as feeder cells (27,28).

Immunization of animals with 741F8 Ab2; generation of an Ab3 response

Once Ab2 were identified they were injected into BALB/c mice as previously described (27,28). Male New Zealand rabbits were injected s.c. with 500µg of purified Ab2 in Freund's complete adjuvant on day 0 and the same amount of Ab2 in Freund's incomplete adjuvant on day 14, followed by two i.m. booster injections in PBS during the next 2 months (27,28).

Cell binding assays

Assays were performed in disposable microfold 96-well microfilter plates. The plate was first treated with 10% fetal calf serum and 1% bovine serum albumin in PBS to block the plates and prevent non-specific binding. Then, 100µl aliquots of 741F8 Ab2 were added to individual wells containing $3x10^5$ viable SK BR 3 cells (in 50µl PBS). 125I-741F8 Ab1 (in 50µl PBS) was then added to each well and the plate shaken for 2 hours at room temperature. After incubation, the plate was washed 3 times with PBS containing 1% bovine serum albumin with suction. The radioactivity in the washed filter paper was measured in a gamma counter (Packard Instruments).

Percent inhibition of the assay was calculated according to the formula

$$\%Inhibition=1-\ (Rt\ )\ /\ (Rmax)\ x\ 100$$

where Rt was the average cpm of the experimental well with 741F8 Ab2 and Rmax was the average maxium binding in the absence of inhibitor (Ab2).

Results

(A) 454C11

Generation of monoclonal anti-idiotype hybridomas

454C11 Ab1 was used to generate Ab2 in BALB/c mice. Three immunizations with 454C11 were required to generate a sufficient Ab2 response in BALB/c mice for fusions. That is, a 1:160 dilution of immune sera produced more than double that of pre-immune sera in binding to the respective Ab1 by Sandwich RIA. Furthermore, immune sera exhibited no binding to KLH or mouse IgG. Culture supernatents of primary fusion cells were initially screened on the basis of their binding to the respective Ab1 and their failure to bind to mouse IgG1, KLH or another unrelated mouse monoclonal Ab1 (mAb 741F8). Of the ~800 fusion wells screened for 454C11 Ab2 production, one Ab2

producing clone was obtained. These cells were grown up by transfering them to larger wells and eventually to a small flask. However, the clone was unstable and declining production of 454C11 Ab2 was observed. This work constitutes the goals of **Specific Aim #1 parts a,b and c** of the original proposal which have been continued into this second year.

(B) 741F8

I. Characterization of Ab2

To assess the reactivity of the anti-idiotype antibody 741F8 Ab2 was tested against a panel of monoclonal antibodies of various specificities belonging to different major Ig subclasses by indirect binding RIA. Results are shown in Figure 1. 741F8 Ab2 bound to its Ab1; there was significant cross reaction with the other two monoclonal antibodies directed against HER/2neu, that is 454C11 and 520C9 which recognize closely related epitopes on HER/2neu to 741F8. Minor cross reaction was seen with the remaining monoclonal antibodies, the most being with MC10, a monoclonal antibody directed against human milk fat globule membrane antigen (HMFG).

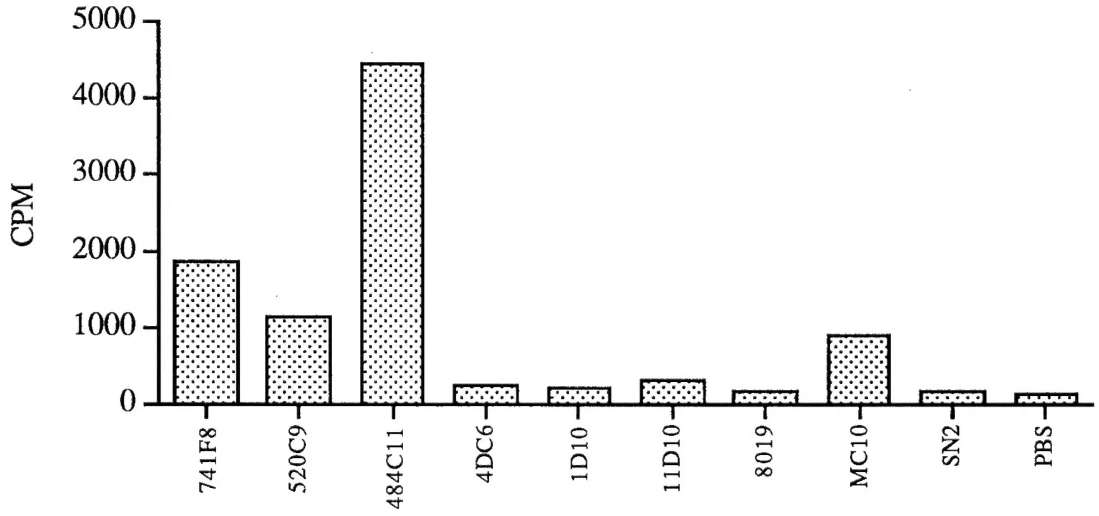

Figure 1. Anti-idiotype specificities of 741F8 (IgG1k). Binding of 741F8 Ab2 to various mouse monoclonal antibodies was determined by indirect RIA.The isotypes of the monoclonal proteins used to coat the plates were 741F8 (IgG1k), 520C9 (IgG1k), 454C11 (IgG2a), 4DC6 (IgG1x), 1D10 (IgG1k), 11D10 (IgG1k), 8019 (IgG1k), MC10 (IgG2a) and SN2 (IgG1k). The results are presented as mean cpm of 3 separate wells. The SD of the data was less than 10% for the assay.

An inhibition study was conducted to determine whether purified 741F8 Ab2 would compete for the binding of a fixed amount of Ab1 to SK-BR-3 cells, which express the tumor antigen HER2/neu. Prior to this, experiments to determine SK-BR-3 cell number and the amount

of radiolabelled 741F8 Ab1 to use were carried out so as to select optimal conditions for the subsequent competitive binding study using purified 741F8 Ab2. Binding of 741F8 Ab1 was linear with respect to cell number (Figure 2a). A cell number of $3x10^5$ SK-BR-3 cells was selected for the subsequent study. SK-BR-3 cells tend to form cell aggregates following their detachment from the cell culture flasks using trypsin, thus using fewer cells would probably lead to larger variation in the inhibition assay as a result. The amount of 741F8 Ab1 ($2x10^5$ cpm) was selected for the inhibition study as amounts above this appeared "saturating" (Figure 2b).

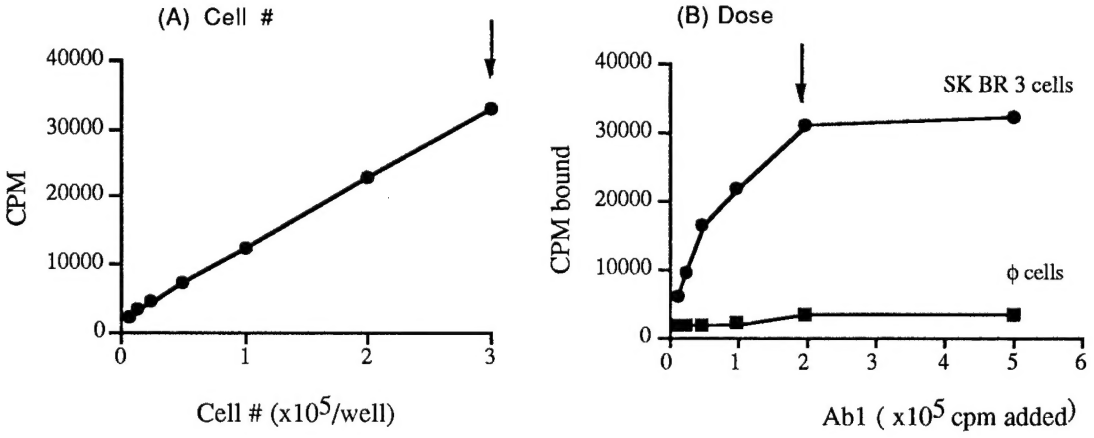

Figure 2.Binding of 741F8 Ab1 to SK-BR-3 cells. (A) Effect of cell number. Different numbers of SK-BR-3 cells were incubated with a fixed amount of radiolabelled 741F8 ($2x10^5$cpm) for 2 h at room temperature with shaking, washed and the filter counted in a gamma counter. (B) Effect of 741F8 Ab1 concentration. Different amounts of radiolabelled 741F8 Ab1 were incubated with $3x10^5$ SK-BR-3 cells for 2h at room temperature with shaking, washed and the filters counted in a gamma counter. The results are presented as the mean cpm of 3 separate wells. The SD of the assay was less than 10% for the assay.

From the inhibition study it appears that as little as ~10ng of 741F8 Ab2 inhibited the binding of radiolabelled 741F8 Ab1 to SK-BR-3 cells by ~50% (Figure 3). No binding was observed with the control human breast cell line, HBL-100, which does not express the tumor associated antigen, NER/2neu on its surface. This work was the goal of **Specific Aim #2a** of the original proposal.

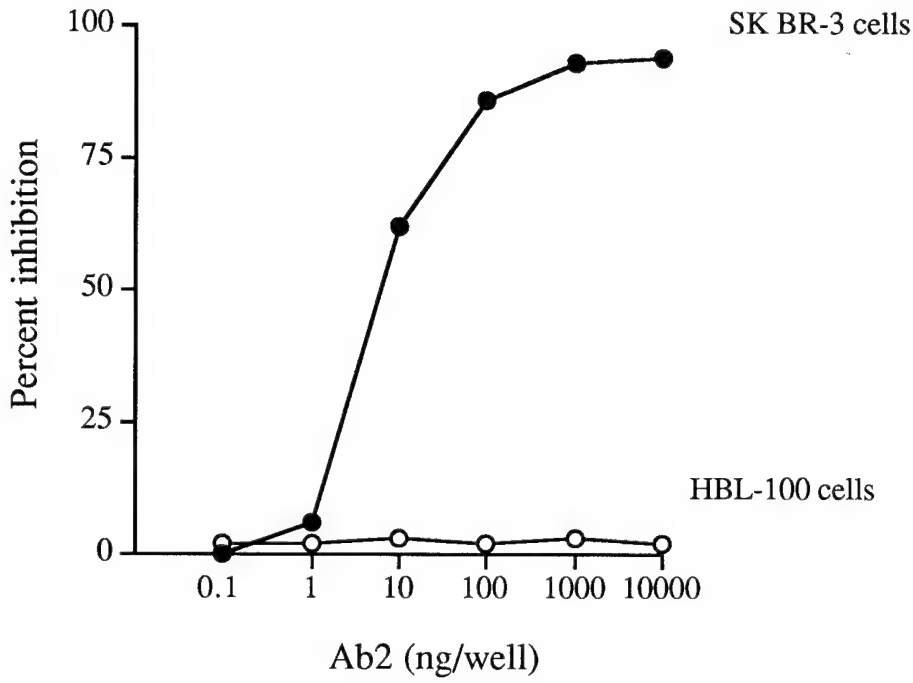

Figure3. Inhibition of 741F8 Ab1 binding to SK-BR-3 cells by purified 741F8 Ab2. Different concentrations of 741F8 Ab2 and a fixed amount of radiolabelled 741F8 Ab1 were added to each well and incubated for 2h at room temperature with shaking with $3x10^5$ SK-BR-3 cells (antigen positive) or HBL-100 cells (control cells).Plates were then washed to remove unbound Ab1 and the filters counted in a gamma counter. Results are the mean percent inhibition of 3 separate wells. The SD was less than 10% for the assay.

II. Generation of 741F8 polyclonal Ab3.

If the Ab2 is a true internal image, then it should induce the production of antigen specific Ab3 response in the absence of exposure to antigen in a genetically unrestricted way and accross species barriers (22,23). To determine this, five BALB/c mice and two rabbits have been immunized with 741F8 Ab2 for the production of Ab3 that might share idiotopes with Ab1 and exhibit identical binding specificity. These studies were the goal of **Specific Aim #2b**.

CONCLUSIONS

(a) Anti-idiotype antibodies from 454C11.

454C11 Ab1 was used to generate Ab2 in female BALB/c mice (**Task #1 of the original proposal**). Despite a high fusion frequency of over 80%, only one well was found to contain Ab2 producing hybridomas. Unfortunately, this clone showed declining Ab2 production when

expanded in a 48 well plate and flasks. Despite rapid cloning of the positive well, no stable Ab2 clones were obtained.

(b) Anti-idiotype antibodies from 741F8.

Studies were conducted to further characterize the 741F8 Ab2 purified from mouse ascites by affinity chromatography using a Hi-Trap protein G column and the purity checked by SDS-PAGE. To assess the specificity of the anti-idiotype antibody, 741F8 Ab2 was incubated with a series of different mouse monoclonal antibodies belonging to different immunoglobulin subclasses by RIA (Figure 1). We found that the specificity of 741F8 Ab2 binding was directed to its respective Ab1 and to the other two monoclonal Ab1's also directed against HER/2neu. Very little binding was observed with 741F8 Ab2 to the other mouse monoclonal Ab1's tested except for MC10, which is also a mouse monoclonal Ab1 directed against HMFG present in malignant breast tissue. This suggests that 741F8 Ab2 contains a public idiotope which is shared amoung various anti-HER/2 neu and anti-breast cancer antigen antibodies. This anti-Id could be a potential reagent for treating a large number of breast cancer patients positive for the above antigens (**Task # 2 of the original proposal**).

Studies were conducted to determine whether purified 741F8 Ab2 exhibited high competitive binding with its respective Ab1 to SK-BR-3 cells, a breast cancer cell line which expresses the antigen HER2/neu to a high degree on its cell surface (**Task #2 of the original proposal**). Experiments to determine optimal conditions for determining percent inhibition of Ab2 binding to SK-BR-3 cells by Ab1 were completed i.e. the number of cells and the amount of Ab1 to add (Figure 2). We established that Ab1 binding to SK-BR-3 cells was linear with respect to cell number over the cell numbers examined. We selected a cell number of $3x10^5$ cells. We then determined the amount of radiolabelled Ab1 to use by incubating these cells with different amounts of radiolabelled 741F8 Ab1. Amounts of $2x10^5$ cpm Ab1 and above appeared to be "saturating" and so we chose to use $2x10^5$ cpm for the inhibition experiments. Using $3x10^5$ cells/well and $2x10^5$ cpm of 741F8 Ab1 we found that ~10ng of purified 741F8 Ab2 inhibited the binding of Ab1 to SK-BR-3 cells by approximately 50% (Figure 3). (**Task#2 of the original proposal).**

Using purified 741F8 Ab2 we plan to determine whether this Ab2 is able to induce the production of antigen specific Ab3 in another group of 5 mice and in another species which we chose to be rabbit (**Task #2 of the original proposal**). Sera from mice and rabbits immunized with 741F8 Ab2 have been collected. We are in the process of conducting specificity experiments with this 741F8 Ab3 sera by looking at the binding of Ab3 to SK-BR-3 cells by flow cytometry. If the 741F8 Ab2 is a true anti-id, it should induce an Ab3 response in both mice and rabbits

and this Ab3 would be expected to selectively bind to SK-BR-3 cells in a way similar to that of its respective Ab1. We then plan to determine the specificity of binding of 741F8 Ab1 and Ab3 to normal human tissues and breast cancer specimens by immunoperoxidase staining. Furthermore, patients peripheral blood mononuclear cells will be isolated by Ficoll density gradient centrifugation. These cells will be cultured *in vitro* with purified 741F8 Ab2. Culture supernatents will be harvested and checked for the production of specific human Ab3. Additionally, we will check the T-cell responses of patients lymphocytes to 741F8 Ab2 by 3H-Thymidine incorporation assay **(Tasks#3 of the original proposal**). Both procedures, that is the immunoperoxidase staining of human tissues and the isolation, culture and T-cell assays are currently used in the labratory for clinical trials in progress with other anti-idiotype antibodies.

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